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ORIGINAL PAPER

Benefits of membrane electrodes in the electrochemistry of metalloproteins: mediated catalysis of *Paracoccus pantotrophus* cytochrome *c* peroxidase by horse cytochrome *c*: a case study

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Abstract A comparative study of direct and mediated electrochemistry of metalloproteins in bulk and membrane-entrapped solutions is presented. This work reports the first electrochemical study of the electron transfer between a bacterial cytochrome *c* peroxidase and horse heart cytochrome *c*. The mediated catalysis of the peroxidase was analysed both using the membrane electrode configuration and with all proteins in solution. An apparent Michaelis constant of 66 ± 4 and 42 ± 5 μM was determined at pH 7.0 and 0 M NaCl for membrane and bulk solutions, respectively. The data revealed that maximum activity occurs at 50 mM NaCl, pH 7.0, with intermolecular rate constants of $(4.4 \pm 0.5) \times 10^6$ and $(1.0 \pm 0.5) \times 10^6$ M^{-1} s^{-1} for membrane-entrapped and bulk solutions, respectively. The influence of parameters such as pH or ionic strength on the mediated catalytic activity was analysed using this approach, drawing attention to the fact that careful analysis of the results is needed to ensure that no artefacts are introduced by the use of the membrane configuration and/or promoters, and therefore the dependence truly reflects the influence of these parameters on the (mediated) catalysis. From the pH dependence, a p*K* of 7.5 was estimated for the mediated enzymatic catalysis.

Keywords Membrane electrode · Mediated catalysis · Bacterial cytochrome *c* peroxidase · Horse cytochrome *c*

Abbreviations

AUME	Gold membrane electrode
BCCP	Bacterial cytochrome *c* peroxidase
ME	Membrane electrode

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Introduction

The use of membrane electrodes (ME), with the protein imprisoned between a dialysis membrane and the electrode surface, has some important advantages. These are (1) the use of very small volumes of protein—2 μl (to overcome the limitation of sample availability), (2) the ease of electrode preparation and low cost, (3) the rapid investigation of various experimental parameters, such as pH and ionic strength and (4) the thin layer configuration, which easily allows quantitative information about the redox processes to be obtained [1, 2].

Moreover, the membranes used in this type of electrode configuration are charged, offering a convenient way to modulate the electric environment close to the electrode surface, and thus ascertain the role of electrostatic interactions in the efficiency of the electron transfer. It is also

important to point out that this configuration is an interesting strategy when adsorption of a protein or an enzyme to the electrode surface is not successfully achieved, hindering the use of protein film voltammetry [3]. Additionally, the entrapment of proteins between the membrane and the electrode surface enables the achievement of thin-layer conditions and avoids diffusional problems [4].

Studies reporting the benefits of the ME in protein electrochemistry and its role in modulating the redox behaviour focused on the direct electrochemistry of small electron transfer proteins. In these works, a critical comparison was made with data obtained with non-ME [5–7]. More recently ME were used to study multiredox cofactor-containing enzymes and their interaction with both physiological and non-physiological redox partners [8, 9]. In both studies, although the direct electrochemistry and the mediated catalysis of the proteins were analysed using that strategy, with the proteins in solution or adsorbed on a modified electrode, no comparison was made among the different strategies.

The mediated catalysis of the bacterial cytochrome *c* peroxidase (BCCP) from *Paracoccus pantotrophus* by *P. pantotrophus* pseudoazurin examined at a gold ME (AUME) was recently reported by us [10]. Cyclic voltammetry was used to analyse the direct transfer to pseudoazurin and to probe its interaction with BCCP. The results obtained showed that this small copper protein is a competent electron donor to BCCP, in agreement with solution steady-state kinetics performed spectrophotometrically [11]. The pH and ionic strength effect on the voltammetric signal of pseudoazurin as well as on the intermolecular rate constant were also easily studied at the ME.

Horse heart cytochrome *c*, a commercially available small *c*-type haem protein, has been previously used as an electron donor to *Paracoccus* BCCP in solution steady-state kinetic studies performed spectrophotometrically [12]. Although it is not a physiological partner of BCCP, this small protein was shown to be kinetically competent as an electron donor to the *Paracoccus* enzyme. However, horse cytochrome *c* exhibits a distinct kinetic and binding behaviour from the physiological partners pseudoazurin, a type I copper protein, and cytochrome c_{550}, which is also a small *c*-type haem protein. The physiological partners bind at the same site on the surface of BCCP near the electron-transferring haem of the enzyme [13], while horse cytochrome *c* has been proposed to have two binding sites, a looser binding site near the electron-transferring haem and an additional tight one in-between the two haems of the enzyme [14]. Below 50 mM NaCl, there is little change in the activity when the electron donor is either cytochrome c_{550} or pseudoazurin. In contrast, when horse cytochrome *c* is used as an electron donor there is a threefold decrease in activity as the ionic strength is lowered, and this correlates with an increased binding affinity [15, 16]. It was concluded that the higher-affinity binding was, in fact, non-productive and that only when that attachment was loosened by raised ionic strength could the horse cytochrome *c* migrate to the true electron transfer site.

This work reports the first electrochemical study of the electron transfer between BCCP and horse cytochrome *c*. The mediated catalysis of BCCP was analysed using the ME configuration and with all proteins in solution. Our aim is to compare the results obtained with these two strategies in order to gain a better insight into the interactions that occur at the ME surface and to highlight the effects of this strategy on the direct and mediated electrochemistry of metalloproteins.

Materials and methods

Proteins and chemicals

Horse heart cytochrome *c* was obtained from Sigma and used without further purification. Pseudoazurin and BCCP were isolated and purified as described before [17]. The concentration of the proteins was determined spectrophotometrically using the extinction coefficient at 409 nm, $\varepsilon = 250\ \text{mM}^{-1}\ \text{cm}^{-1}$, and at 550 nm, $\varepsilon = 29.5\ \text{mM}^{-1}\ \text{cm}^{-1}$, for fully oxidized BCCP and reduced horse cytochrome *c*, respectively [17, 18].

4,4′-Dithiodipyridine and $CaCl_2$ were obtained from Sigma and all other chemicals were pro analysis grade. All solutions were prepared with deionized water from a Milli-Q water purification system.

Apparatus and procedures

The cyclic voltammograms were obtained using an EG&G-PAR model 273A potentiostat/galvanostat controlled via the 270 software. The scan rate varied between 5 and 200 mV s^{-1}. Throughout this article, all potential values are referred to the standard hydrogen electrode.

A conventional three-electrode configuration cell was used, with a platinum auxiliary electrode and an Ag/AgCl reference electrode (BAS MF-2052; 205 mV vs. the standard hydrogen electrode). The working electrode was a gold disk electrode from BAS (MF-2014) with a nominal radius of 0.8 mm. The effective surface area of the electrode was determined from its response in a known concentration of the ferrocyanide/ferricyanide couple ($D = 7.84 \times 10^{-6}\ \text{cm}^2\ \text{s}^{-1}$ [19]) and was found to be 0.0195 cm^2.

Before each experiment the electrode was polished by hand on a polishing cloth (Buehler 40-7212) using a water/alumina (0.05 μm) slurry (Buehler 40-6365-006),

sonicated for 5 min, rinsed well with Milli-Q water and finally dipped into 1 mM 4,4′-dithiodipyridine solution for 5 min. The membrane configuration was prepared as previously described [6] using a negatively charged Spectra/Por MWCO 3500 membrane.

In typical experiments, the supporting electrolyte, as well as the working solution, contained 10 mM phosphate buffer pH 7.0 ± 0.1, 0.5 mM 4,4′-dithiodipyridine and 1 mM $CaCl_2$. Horse cytochrome *c* was present in a concentration of 100 μM and the concentration of BCCP varied between 0.25 and 1 μM. In the experiments with a saturating concentration of substrate, 100–350 μM H_2O_2 was present in the electrolyte solution. The effect of substrate concentration was determined by varying the H_2O_2 concentration between 10 and 200 μM. The pH of the electrolyte was varied from 5.4 to 10.5 by adding 5 M HCl or 2 M NaOH to a mixed-buffer system containing 10 mM 2-morpholinoethanesulfonic acid, sodium phosphate, *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid, tris(hydroxymethyl) aminomethane and 3-cyclohexylamino-1-propanesulfonic acid. The effect of the ionic strength was studied by adding increasing amounts of NaCl (up to 500 mM).

It should be pointed out that in order to have reproducible results for some BCCP concentrations more than one ME had to be prepared, to cover the whole range of experimental variables. This is due to the fact that the enzyme exists in a monomer–dimer equilibrium in which only the dimer is active. Dilution of the enzyme shifts the equilibrium towards the monomer and therefore the activity is lost with time [14], which corresponds to a decrease of the catalytic current.

All solutions were deaerated for 10 min with high-purity nitrogen, and all measurements were performed at least in duplicate in a temperature-controlled room at 20 ± 1 °C.

Results and discussion

Catalytic activity of *P. pantotrophus* BCCP with horse heart cytochrome *c* as an electron donor

ME cyclic voltammetry

The electrochemical behaviour of horse cytochrome *c* is well known either in bulk or entrapped on a ME. Indeed, Eddowes and Hill [20] found that essentially reversible voltammetry of cytochrome *c* in solution could be observed at a 4,4′-dipyridyl-modified gold electrode. Latter, Lojou and Bianco [5] showed that fast electrochemical response was observed when a thin layer of protein solution was entrapped between a negatively charged dialysis membrane and a non-modified gold electrode surface. In both cases favourable conditions for electron transfer to occur were achieved, which accounts for the similarity of the values determined for the formal potentials.

In this work, the direct electrochemistry of cytochrome *c* was revisited at a AUME, but this time in the presence of 4,4′-dithiodipyridine (data not shown). The promoter was used in order to have similar experimental conditions for the studies both in solution and with the membrane-configuration electrode (further reasons will be explained below).

Thin-layer behaviour will be observed as long as the entrapped layer thickness l is smaller than the diffusion-layer thickness for a given experimental time scale, t:

$$l < (2Dt)^{1/2} \quad (1)$$

where D is the diffusion coefficient of the species [21]. Under our experimental conditions (cyclic voltammetry with scan rates between 5 and 200 mV s^{-1}), this condition was verified for the lowest scan rates ($v < 100$ mV s^{-1}), with i_p varying linearly with v in this range. From this variation [21], an entrapped-solution volume, V, of 1.2×10^{-5} cm^3 was estimated, which corresponds to an entrapped-layer thickness $l = V/A = 6.2 \times 10^{-4}$ cm. Since the diffusion coefficient of cytochrome *c* is $D = 1.2 \times 10^{-6}$ cm^2 s^{-1} (see below), thin-layer conditions occur for $v < 160$ mV s^{-1}, as verified.

The ratio of the cathodic (i_{pc}) and anodic (i_{pa}) peak currents was close to 1 and the peak-to-peak separation, $\Delta E_p = E_{pa} - E_{pc}$, was close to 20 mV for the lowest scan rates. In the same range, the width at half height ($\Delta E_{p,1/2}$) was also constant for the cathodic and anodic peaks, with a value close to 90 mV. Although ΔE_p increased with v, the average of the reduction and oxidation peak potentials remained almost constant for all scan rates and a formal reduction potential $E^{\circ\prime} = (E_{pc} + E_{pa})/2 = 250 \pm 5$ mV was estimated at pH 7.0, in agreement with other reported values (e.g. 280 mV, ME at pH 7.6 [5], and 255 mV, bulk solution at pH 7.0 [20]).

The catalytic activity of *P. pantotrophus* BCCP towards horse cytochrome *c* was then investigated at the AUME, with working solutions of 100 μM cytochrome *c* and different BCCP concentrations (0.25–1 μM) in the presence of a saturating substrate concentration (125–350 μM, depending on the enzyme concentration) or with a working solution of 100 μM cytochrome *c* and 0.25 μM BCCP in the presence of increasing H_2O_2 concentrations. These solutions and the electrolyte contained 1 mM $CaCl_2$ (for enzyme activation [12, 22]).

The cytochrome *c* response is unchanged in the presence of BCCP. Moreover, although cytochrome *c* is known to feature a small peroxidase activity [23, 24], its response also remained unchanged upon addition of H_2O_2 in the absence of enzyme. However, it is clear from Fig. 1a that in the presence of BCCP the cytochrome *c* peak current

increases with H_2O_2 concentration in the electrolyte, and a sigmoidal wave develops when a saturating concentration of substrate is reached. Voltammograms from solutions containing only BCCP either in the presence or in the absence of H_2O_2 were indistinguishable from the background current (data not shown).

The half-wave potential of the sigmoidal waves ($E_{1/2} = 275 \pm 10$ mV $= E^{o\prime}$) shows that the actual transfer process is the catalysed reduction of cytochrome *c*. The catalytic current is scan rate independent up to 50 mV s^{-1} and increases for increasing BCCP concentrations. This behaviour is consistent with a reaction mechanism involving an initial heterogeneous electron transfer reaction at the electrode (Fig. 2, step 1), followed by homogeneous chemical reactions: the oxidized form of cytochrome *c* is regenerated by BCCP (Fig. 2, step 2) which, in turn, is recycled by H_2O_2 (Fig. 2, step 3). This mechanism can be simplified to

$$\begin{aligned} &\mathrm{Ox} + e^- \rightleftharpoons \mathrm{Red} \\ &\mathrm{Red} + Z \rightarrow \mathrm{Ox} \end{aligned} \quad (2)$$

provided that the following conditions are obeyed: (1) the heterogeneous electron transfer (Fig. 2, step 1) is a reversible reaction; (2) the homogeneous chemical reaction (Fig. 2, step 2) is irreversible; (3) the reaction between cytochrome *c* and BCCP is pseudo first order with a reaction rate constant given by $k' = kC_{\mathrm{BCCP}}$, where *k* is the second-order rate constant [10, 25].

The first condition is well obeyed, as previously demonstrated. The second condition is fulfilled because BCCP is being reoxidized in the catalytic cycle owing to the excess of H_2O_2 in solution (Fig. 2, step 3), and not by transferring electrons back to horse cytochrome *c*. As to requisite 3, it would imply that BCCP is present in large excess, which is not the case. However, under saturating concentrations of H_2O_2, this requisite is obeyed when the rate of recycling the oxidised BCCP by H_2O_2 is not rate-limiting. If this is the case, BCCP will always be available to react with cytochrome *c* and pseudo-first-order conditions are met.

Cyclic voltammograms at 20 mV s^{-1} were obtained for 100 μM cytochrome *c* and increasing BCCP concentrations (0.25–1 μM), in the absence and in the presence of a saturating concentration of H_2O_2 (125–350 μM, depending on the enzyme concentration), and are shown in Fig. 3.

From these recordings the catalytic current (i_{cat}) was determined as the difference between the current in the presence and in the absence of substrate, both measured at the same potential. The data was treated according to Laviron's theory for diffusionless electrochemical systems [4, 10], where the catalytic current is given by

$$i_{\mathrm{cat}} = n F k' V C_{\mathrm{Cyt}c} \quad (3)$$

In this equation, *V* is the entrapped-solution volume, *C* is the concentration and the other terms have the usual meaning.

From the variation of the pseudo-first-order rate constant, *k*′, with BCCP concentration, an intermolecular rate

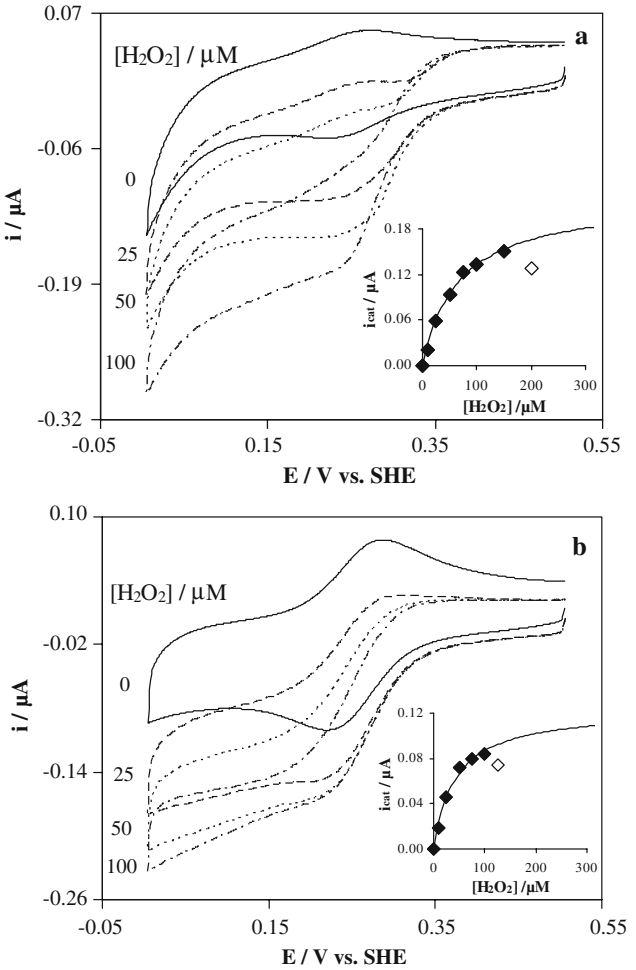

Fig. 1 Cyclic voltammograms ($v = 20$ mV s^{-1}) at the gold membrane electrode (**a**) or bulk solution (**b**) of 100 μM horse heart cytochrome *c* and 0.5 μM bacterial cytochrome *c* peroxidase (BCCP) in the presence of increasing concentrations of H_2O_2. The medium consisted of 10 mM phosphate buffer pH 7.0, 0.5 mM 4,4′-dithiodipyridine and 1 mM $CaCl_2$. *SHE* standard hydrogen electrode

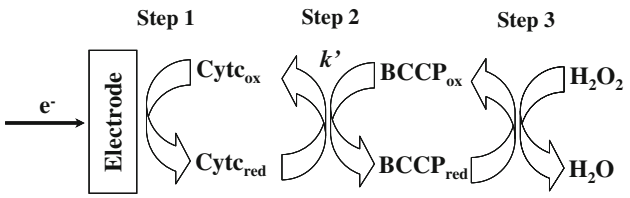

Fig. 2 Mediation scheme for BCCP: the electrode reduces horse heart cytochrome *c*, which is immediately reoxidized by BCCP; the level of oxidized BCCP is then restored by conversion of hydrogen peroxide to water

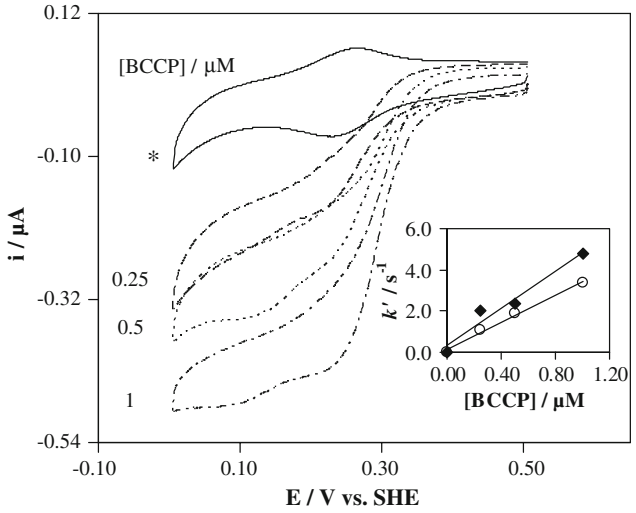

Fig. 3 Cyclic voltammograms ($v = 20$ mV s^{-1}) obtained at the gold membrane electrode for 100 μM cytochrome *c* and increasing BCCP concentrations (0.25–1 μM), in the absence (*asterisk*) and in the presence of a saturating H_2O_2 concentration (125–350 μM, depending on the enzyme concentration). *Insert*: Variation of the pseudo-first-order rate constant with BCCP concentration for 0 mM (*circles*) and 50 mM (*diamonds*) NaCl. The medium consisted of 10 mM phosphate buffer pH 7.0, 0.5 mM 4,4′-dithiodipyridine and 1 mM $CaCl_2$

constant $k = (3.2 \pm 0.5) \times 10^6$ M^{-1} s^{-1} was estimated (Fig. 3, insert, circles).

For assays where the H_2O_2 concentration was varied, for a constant cytochrome *c* and BCCP mixture, the catalytic current was plotted as a function of the substrate concentration (Fig. 1a, insert). It is clear that the catalytic current decreases after 150 μM H_2O_2 and so the data were fitted to the Michaelis–Menten kinetics for concentrations up to that value. Since thin-layer conditions are verified, the Michaelis–Menten equation has the form

$$i_{cat} = \frac{i_{max} C_{H_2O_2}}{C_{H_2O_2} + K_M} = \frac{nFk'VC_{Cytc}C_{H_2O_2}}{C_{H_2O_2} + K_M} \quad (4)$$

Using the CERN library Fortran program MINUIT algorithm, we estimated an apparent Michaelis–Menten constant $K_M = 66 \pm 4$ μM and an intermolecular electron transfer rate constant $k = (3.4 \pm 0.3) \times 10^6$ M^{-1} s^{-1}.

This electron transfer rate constant is higher than the value determined for a physiological donor, pseudoazurin ($(1.4 \pm 0.2) \times 10^5$ M^{-1} s^{-1} [10]). The more likely explanation for the difference between the rate constants would be a difference in the redox potential of these two electron donors, but the values determined at pH 7.0 are very similar.

Therefore, this behaviour must be related to another factor that influences the donor–enzyme interaction. In the case of these small electron donors the global charge surface is high and asymmetrically distributed, creating a significant dipole moment. Actually, the dipole moment of pseudoazurin is 651 D, and that of horse cytochrome *c* is 299 D [11]. This twofold difference in the dipole moment probably results in a stronger association of pseudoazurin with BCCP, and consequently the dissociation of the complex is disfavoured, explaining the lower rate constants observed.

Solution cyclic voltammetry

The mediated catalysis of BCCP by horse cytochrome *c* was also analysed at the gold electrode from solutions containing 100 μM horse heart cytochrome *c*, 0.5 μM BCCP and 1 mM $CaCl_2$. Again, the peak current increases with H_2O_2 concentration in the electrolyte, and a sigmoidal wave develops when a saturating concentration of substrate (100 μM) is present in the electrolyte, which is scan rate independent up to 50 mV s^{-1} (Fig. 1b).

The theory describing such a mechanism for diffusion-controlled processes was developed by Nicholson and Shain [26] and applied to several kinetic studies of reactions between mediators and redox proteins [25]. The intermolecular rate constant can also be calculated from the value of the H_2O_2 saturated limiting current using Eq. 5 [27, 28]:

$$i_{cat} = nFAD^{1/2}C_{Cytc}(k')^{1/2} \quad (5)$$

In this equation, *D* is the diffusion coefficient of cytochrome *c*. The valid value for our experimental conditions, $D = (1.2 \pm 0.1) \times 10^{-6}$ cm^2 s^{-1}, was computed from the dependence on the scan rate of either the cathodic or the anodic peak currents of the cyclic voltammograms for solutions containing both cytochrome *c* and BCCP in the absence of H_2O_2, using the Randles–Ševčík equation [21]. Calculations using Eq. 5 gave an intermolecular rate constant of $k = (4.0 \pm 0.5) \times 10^5$ M^{-1} s^{-1}.

The catalytic current for the different H_2O_2 concentrations (Fig. 1b, insert) was also fitted to Michaelis–Menten kinetics for concentrations up to 100 μM, and $K_M = 42 \pm 5$ μM and $k = (3.9 \pm 0.3) \times 10^5$ M^{-1} s^{-1} were obtained.

Similar apparent Michaelis–Menten constants were estimated for the proteins in bulk and entrapped solution, indicating that the membrane does not hamper the substrate diffusion. A higher value for the intermolecular rate constant was obtained for the proteins using the membrane configuration, reflecting a more favourable domain for the molecular interactions that must be established for electron transfer to occur between BCCP and cytochrome *c*. In both situations an inhibition effect by the substrate is observed for higher H_2O_2 concentrations, as verified in the case of the catalysis mediated by pseudoazurin [10]. A similar

inhibitory effect was observed in the study of other peroxidases [29–31].

Ionic strength and pH effects

One of the advantages of the ME approach is the ability to rapidly investigate effects on the redox behaviour of various experimental conditions, such as the pH or the supporting electrolyte composition. Another advantage of the approach is the small amount of protein needed for a series of measurements. This is so because once the ME has been mounted in the cell, it does not need to be removed or exchanged while changing any of those variables.

The same approach can also be applied to study the effect of such experimental variables on the kinetic activity of a more complex system, such as in mediated catalysis. Changes in the pH and/or the ionic strength of the medium may influence the rate of association, pre-orientation within the encounter complex, stability of the reactive complex or/and rate of dissociation of the products. However, the interpretation of ionic strength (or pH) dependences of the kinetic activity is complex and the behaviour observed should not be masked by the influence of those variables on the direct electrochemistry of the redox partners.

The effect of the ionic strength on the intermolecular rate constant for BCCP and horse cytochrome *c* was analysed with the proteins entrapped at the AUME and in bulk solution with a working solution containing 100 μM horse cytochrome *c*, 0.5 μM BCCP and 1 mM $CaCl_2$, in the presence of increasing amounts of NaCl (in the range 0–500 mM) and a saturated H_2O_2 concentration.

In both situations, the ionic strength dependence of the mediated catalysis has a bell-shaped curve, with a maximum activity at 50 mM NaCl (Fig. 4). The decrease of activity with increasing NaCl concentrations is consistent with the electrostatic character of the interaction between BCCP and the electron donor, cytochrome *c*. Indeed, cytochrome *c* has an asymmetric charge distribution with a positive surface that surrounds the exposed haem edge, which is the region proposed to interact with the negative surface around the exposed electron-transferring haem of the peroxidase [12, 14]. The increase of activity at low NaCl concentrations can be explained considering that for the lowest ionic strength the encounter complex formed has an orientation that is not so favourable for electron transfer. Therefore, the increase in NaCl concentration enables the lateral search at the peroxidase surface for the competent electron transfer orientation [11, 32]. Another explanation for this effect is that at low ionic strength cytochrome *c* reduction at the electrode might be affected by the lower k_{off} rate constant of the complex with BCCP.

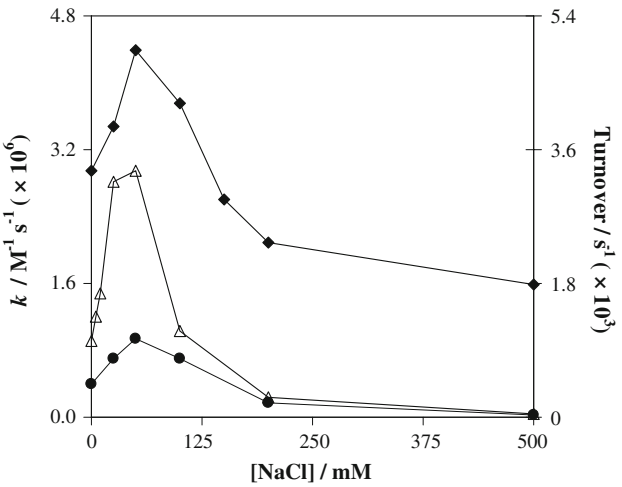

Fig. 4 Effect of ionic strength on the intermolecular rate constant for 100 μM horse heart cytochrome *c*, 0.5 μM BCCP and saturating H_2O_2 (150 μM *diamonds* or 100 μM *circles*) at the gold membrane electrode (*diamonds*) or in bulk solution (*circles*) and on the activity of BCCP with horse cytochrome *c* as an electron donor from solution steady-state kinetics performed spectrophotometrically (*triangles*) [11]. The medium consisted of 10 mM phosphate buffer pH 7.0 and 1 mM $CaCl_2$

Using the approach described above (Eq. 3), one can use the analysis of the catalytic current variation as a function of BCCP concentration (Fig. 3, insert, diamonds) to estimate an intermolecular rate constant for maximum activity $k = (4.4 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$ for the membrane configuration. In the case of the bulk solution, a rate constant for maximum activity $k = (1.0 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$ was determined using Eq. 5.

Similar behaviour was observed by solution steady-state kinetics performed spectrophotometrically, when this small cytochrome is used as an electron donor, with a maximum turnover also occurring at 50 mM NaCl [11], as can be observed in Fig. 4. However, in the voltammetric experiments with the membrane the variation in activity with ionic strength is less pronounced than that found with the proteins in bulk solution (either in the voltammetric assays or in solution steady-state kinetics performed spectrophotometrically). In fact, from 50 to 500 mM NaCl the activity decreases by 64% at the ME, while in the other two cases the decrease is 98%.

A similar effect was observed for the mediated catalysis of *P. pantotrophus* BCCP by *P. pantotrophus* pseudoazurin, where similar profiles were obtained with the membrane configuration and solution steady-state kinetics performed spectrophotometrically, though the maximum activity was observed for higher NaCl concentration in the former case. The differences may be due to the charge of the membrane, which somehow favours the formation of the encounter complex, making it less dependent on the ionic strength, in spite of the electrostatic forces that govern the interaction.

This may be also the reason why higher rate constants were determined for horse cytochrome *c* using the membrane configuration, although values of the same order of magnitude were obtained for maximum activity [$k = (4.4 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$ for the membrane configuration and $k = (1.0 \pm 0.5) \times 10^6\ M^{-1}\ s^{-1}$ for bulk solution] as illustrated in Fig. 4.

The behaviour observed in the voltammetric experiments truly reflects the effect of ionic strength on the interaction between the enzyme and the electron donor rather than on the direct electrochemistry of horse cytochrome *c* due to the presence of the promoter. The horse cytochrome *c* peak current was shown to be NaCl-independent when 4,4′-dithiodipyridine is present in the medium (data not shown). Behaviour different from that observed at a bare gold electrode in the membrane configuration was evidenced, where a sharp decrease in current was observed at increasing NaCl concentrations [5]. Similar behaviour was also observed for the direct electrochemistry of pseudoazurin at a dithiodipyridine gold modified electrode [10].

The pH behaviour of horse cytochrome *c* is well known to present a p*K* close to 9.5 associated with the disappearance of the 695 nm absorption band, which is believed to be due to the replacement of the iron-coordinated methionine residue by a nitrogenous ligand, assigned as Lys73 [33, 34].

Previous bulk voltammetric studies of horse cytochrome *c* at a 4,4′-bipyridyl-modified gold electrode in the pH range 4.5–10.5 showed that while the peak potential remains essentially constant until pH 9, the peak current profile has a bell shape [35]. The maximum current was observed at pH 7 and two p*K* values around 9 and 5 were computed from that variation. The former is in agreement with the value obtained by other techniques, as well as with the observed E_p variation. The latter was attributed by the authors to the protonation of the promoter that disrupts the interaction between the gold surface and the protein, leading to a decrease of the electrochemical current [35].

For the reasons presented above, the pH dependence of the intermolecular rate constant for BCCP and horse cytochrome *c* was analysed at the bare AUME, i.e. in the absence of 4,4′-dithiodipyridine.

In order to accurately interpret the results of this pH dependence, the effect of this parameter on the electrochemical signal of horse cytochrome *c* alone was studied between pH 5 and 10 in the same conditions. As expected, the redox potential remained constant for pH < 9 and a pronounced decrease was observed for higher pH values (data not shown). A p*K* of 9.1 ± 0.1 was determined, in accordance with reported values [33–35]. For the peak current, a bell-shaped profile was obtained (Fig. 5, circles), behaviour similar to that observed for bulk solution in the

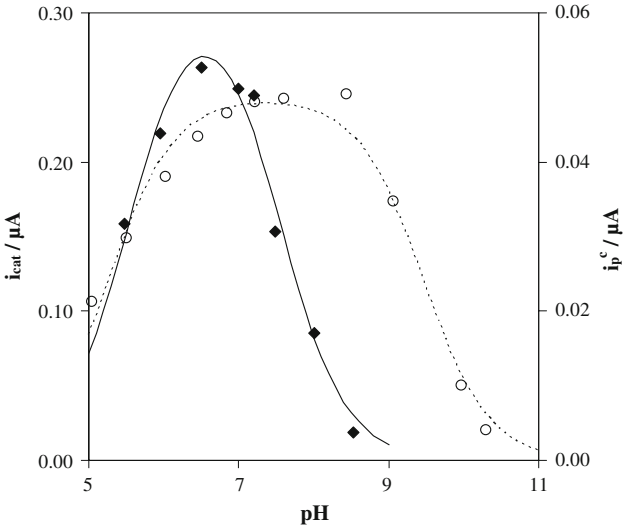

Fig. 5 pH dependence of 100 μM horse heart cytochrome *c* at the gold membrane electrode (*circles*) and catalytic current for 100 μM horse heart cytochrome *c*, 1.0 μM BCCP and saturating H_2O_2 (350 μM) at the gold membrane electrode (*diamonds*). The medium consisted of a mixed-buffer system (10 mM each) and 1 mM $CaCl_2$. The curves were fitted with Eq. 6

presence of the promoter [35]. The peak current pH dependence was fitted to the following equation [36]

$$i_{cat} = \left(i_{opt}\right) \Big/ \left(1 + 10^{(pKa_1 - pH)} + 10^{(pH - pKa_2)}\right) \quad (6)$$

and p*K* values of 5.3 ± 0.3 and 9.5 ± 0.3 were estimated. The latter is in clear agreement with the characteristic alkaline p*K* of this protein.

Several electrochemical studies using different approaches have observed a similar behaviour for the dependence on the pH of the horse cytochrome *c* current, and in some cases an acidic p*K* with an identical value, within the experimental errors, has been determined. However, depending on the authors and the conditions employed in the studies, the p*K* was assigned differently, namely to the proton-dissociation constant of the promoter [35], the Coulombic properties of the electrode surface [37, 38], the competitive adsorption of protons on the clay-modified electrodes [39] or the protein denaturation induced by the applied electric field at the electrode–solution interface [40]. In the present work a ME was employed, and so another possible cause for the profile observed at low pH is the degradation of the membrane–protein–electrode interaction, given that in the absence of a promoter charge effects stand out as being most important, as previously mentioned.

In the present study the same behaviour was observed either in the presence (data not shown) or in the absence of 4,4′-dithiodipyridine, eliminating the promoter protonation as the possible cause. Moreover, considering the fact that

the profile obtained at the bare ME is similar to that observed for the several bulk conditions, it seems that the acidic p*K* is not due to an artefact of the membrane.

In fact, this acidic p*K* may be the result of an acidic residue or a group of residues involved in the interaction with the electrode, but not affecting the haem environment and consequently not changing the redox potential. Denaturation of the protein would also lower the concentration of horse cytochrome *c* responding at low pH, and must be considered. Indeed, the analysis of the voltammograms of horse cytochrome *c* at decreasing electrolyte pH shows that the redox signal is at the same potential, but with a decreasing current which does not return to the initial value when the pH is raised back to 7.0 (data not shown).

The pH effect in the mediated catalysis at the AUME was studied in the range 5–10, using solutions containing 100 μM horse cytochrome *c*, 1 μM BCCP, 1 mM $CaCl_2$ and a saturated H_2O_2 concentration (350 μM). The catalytic current presents a bell-shape profiled (Fig. 5, diamonds) with no activity being observed above pH 9, which can be attributed to the inactivation of the enzyme at high pH [10]. This curve can be fitted to Eq. 6 with i_{opt} at pH 6.5 and two p*K* values, 5.6 ± 0.1 and 7.5 ± 0.1.

Taking into account the above results, we cannot conclude if the acidic p*K* estimated for the mediated catalysis is also related to the protein–protein interaction or only due to horse cytochrome *c* alone. However, preliminary solution steady-state kinetics performed spectrophotometrically of *Paracoccus* BCCP using horse cytochrome *c* as an electron donor (Pauleta et al., unpublished results) revealed an acidic p*K* at the same value, and similar values were observed for the enzymatic activity of *Pseudomonas aeruginosa* (5.2) [41] and *Rhodobacter capsulatus* (6.1) [42] BCCP.

Moreover, binding studies using microcalorimetry showed that the formation of this electron transfer complex involves the release of 0.46 protons at pH 6.0, which also suggests the existence of an acidic p*K*, at around pH 6.0 [16]. Therefore, this p*K* reflects a proton-dissociation process associated both with the horse cytochrome *c* and with the interaction/activity of this protein with BCCP.

A more basic p*K* was also observed for the solution steady-state kinetics determined spectrophotometrically of *P. aeruginosa* (6.8) and *R. capsulatus* (7.9) BCCP [41, 42], as now estimated for the *Paracoccus* enzyme. This p*K* can be clearly attributed to the mediated enzymatic catalysis, since the current profile of the horse cytochrome *c* itself is invariable in this pH region (Fig. 5). As several authors have suggested, these p*K* values can be assigned to the propionates of the BCCP haems, histidine ligands or proposed residues involved in the catalysis such as Glu128 [32, 42, 43]. However, the identity of the residue(s) responsible for this proton dissociation can only be disentangled through site-directed mutagenesis.

Conclusions

This work highlights the use of ME in the study of direct and mediated electrochemistry of metalloproteins. The results reported in the present work show that ME are very attractive for the study of complex metalloproteins systems, particularly with regard to the very small amounts of protein needed and the ease with which experimental variables can be changed.

Mediated catalysis of BCCP by horse cytochrome *c* was easily analysed, with minimum consumption of proteins, just varying the H_2O_2 concentration in the electrolyte solution. While the proteins stay entrapped in close vicinity to the electrode surface, small ions can diffuse through the membrane. For the same reasons, the effect on the catalytic activity of experimental conditions, such as pH and ionic strength, can be rapidly analysed.

The fitting to Michaelis–Menten kinetics of the experimental data, from studies with the proteins either entrapped in the membrane or freely diffusing in solution, led to similar K_M values, showing that no artefacts are introduced in the study by the use of a ME. Higher values for the intermolecular rate constants for horse cytochrome *c* and BCCP were obtained with the membrane configuration, which shows that the membrane does not disrupts the formation of the encounter complex and can even favour it. Since electrostatic interactions are fundamental for the formation of the complexes between *P. pantotrophus* BCCP and its electron donors, this configuration seems to be an adequate option for the electrochemical study of the mediated catalysis in biological systems, where electrostatic forces play an important role.

To draw conclusions about the influence of variables, such as pH or ionic strength, on the mediated catalytic activity, one must first pay attention to the behaviour of the mediator, i.e. the protein that actually exchanges the electrons with the electrode. For this reason, the ionic strength effect was analysed using a promoter to render the cytochrome *c*–electrode–membrane interaction more hydrophobic in nature, so the variation observed for the catalytic activity could only be due to changes in the binding between BCCP and the redox partner. On the other hand, the use of promoters might mask the pH effect depending on their acid–base behaviour and a possible interference must be verified.

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